bio-protocol

Published: Jun 20, 2022

Protocol for High Throughput Screening of Antibody Phage Libraries

Vanshika Singh[1], Sonal Garg[1], Nisha Raj[1], Asha Lukose[1], Deepti Jamwal[1], Reshma Perween[1], Samridhi Dhyani[1], Hilal Ahamed Parray[1], Chandresh Sharma[1] and Rajesh Kumar[1, 2, *]

[1]Translational Health Science & Technology Institute, NCR Biotech Science Cluster, Faridabad, Haryana-121001, India
[2]Institutes of Advanced Virology, Bio 360 Life Science Park, Trivandrum, Kerala
*For correspondence: rajesh@iav.res.in; rajesh@thsti.res.in

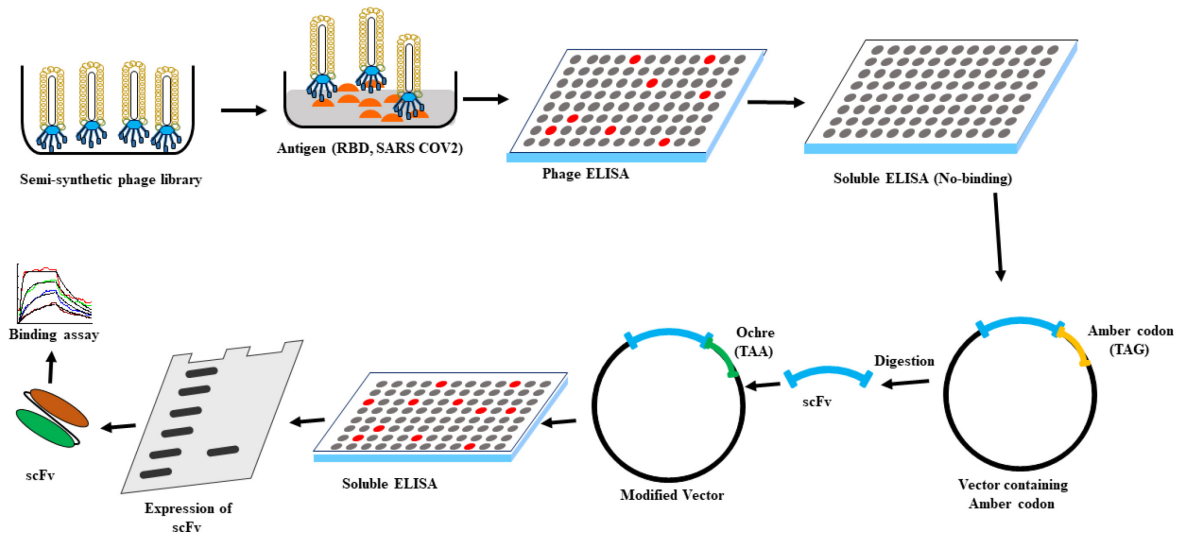
Abstract

Phage display is a proven and widely used technology for selecting specific antibodies against desired targets. However, an immense amount of effort is required to identify and screen the desired positive clones from large and diverse combinatorial libraries. On the other hand, the selection of positive binding clones from synthetic and semi-synthetic libraries has an inherent bias toward clones with randomly produced amber stop codons, making it more difficult to identify desirable binding antibodies. To overcome the screening of desired clones with amber codons, we present a step-by-step approach for effective phage library screening to isolate useful antibodies. The procedure calls for creating a simple new vector system for soluble production of phage ELISA positive binding clones with one or more amber stop codons in their single-chain antibody fragment (scFv) gene sequences, which is otherwise difficult in standard screening.

Keywords: Phage display, Amber codon, scFv, High-throughput screening, Novel vector system

This protocol was validated in: Biotechnol Prog (2021), DOI: 10.1002/btpr.3136

Graphical abstract:



Background

Biomolecules based on monoclonal antibodies are commonly utilized for disease detection and prevention (Borghardt *et al*., 2018; Parray *et al*., 2020; Kumar *et al*., 2022). The single-chain variable fragment (scFv) antibody is one of the most often exploited biomolecules because it is the smallest antibody unit and has low immunogenicity and low-cost production properties (Kumar *et al*., 2019b; Parray *et al*., 2020). The scFv is the most commonly employed combinatorial therapeutic entity, either alone or in combination with other medications (Frenzel *et al*., 2016; Kumar *et al*., 2019b). An antibody in the form of scFv has variable heavy (VH) and light-chain (VL) sections that are linked by an efficient linker that can be effectively produced in *E. coli* (Kumar *et al*., 2012; Kumar *et al*., 2019a). The phage display technique is the most popular and successful way of generating scFv antibody fragments among all *in vitro* display methods. The size and functional diversity of the library used for screening enhances the efficiency of isolating scFv molecules from phage display antibody libraries. The most common issue with the soluble expression of scFv clones from phage libraries is the higher frequency of amber codons within the scFv gene, resulting in the premature expression of scFv clones in non-suppressor *E. coli* strains. This is more common in the case of synthetic and semi-synthetic libraries because these libraries are constructed randomly at few residues—particularly at NNN, NNK, NWG, NWC, and NSG codons—which increase the biased inclusion of amber codons (Marcus *et al*., 2006). Due to the frequent presence of amber codons within antibody gene sequences, the isolation of functional soluble scFv molecules is the most prevalent problem encountered during the screening of synthetic and semi-synthetic libraries, resulting in premature expression of scFv clones in non-suppressor *E. coli* strains (Barderas *et al*., 2006; Perween *et al*., 2021a). However, the inclusion of an amber stop codon does not affect the display of scFvs on the phage surface in *E. coli* suppressor strains, but it reduces the overall yield in terms of the total number of functional soluble scFv protein-expressing clones.

Directing individual scFv genes to be resynthesized or using Kunkel mutagenesis are two popular ways to solve this problem. Both of these processes become expensive and time demanding, considering when the purpose is to screen a substantial proportion of clones, especially in viral targets where a large amount of screening is essential to generate a small number of neutralizing clones (Reader *et al*., 2019).

In this Bio-protocol, we describe a novel strategy for rapid screening of scFvs containing amber codons and turning them into usable soluble scFvs that can be applied to several phage antibody libraries. We discuss a fast and

reliable screening strategy that can be used to screen a large number of phage antibody libraries with amber stop codons (TAG) in the encoding series.

Materials and Reagents

All chemicals are of Analytical Reagent Molecular Biology/Tissue culture grade.

PRODUCT NAME	CATALOGUE NUMBER	COMPANY NAME
(3-(N-morpholino) propane sulphonic acid) MOPs	M1254	Sigma-Aldrich
2× YT media	G034-500G	Himedia
Absolute ethanol	24102	Sigma-Aldrich
Acetic acid	W200603-1KG-K	Sigma-Aldrich
Acrylamide	A8887-100G	Sigma-Aldrich
Agarose	MB080-100G	Himedia
Alkaline Phosphatase Blue Membrane substrate solution	AB0300	Sigma-Aldrich
Ampicillin	SD002	Himedia
Anti-rabbit HRP	Code: 111-035-144	Jackson Immune Research
Beta-mercapto ethanol	21985023	ThermoFisher
Bis-Acrylamide	A2792-100ml	Sigma-Aldrich
Boric acid	MB007	Himedia
Bovine Serum Albumin (BSA)	A3059-10G	Sigma-Aldrich
Bright-Glo Luciferase assay system	E2610	Promega
Bromophenol Blue	B0126-25G	Sigma-Aldrich
Calcium chloride	GRM710	Himedia
Cut smart buffer	B6004S	New England Biolabs
Cyclosporine	RM8155	Himedia
DEAE-dextran	MB145	Himedia
Diethanolamine	RM8218	Himedia
Diethyl pyro carbonate (DEPC)	D43060	RPI – Research Products International
Dimethyl sulphoxide (DMSO)	673439	Sigma-Aldrich
DpnI enzyme	R0176S	New England Biolabs
Dulbecco's Modified Eagle Medium (DMEM)	11965118	Gibco™
Ethylene diamine tetra acetic acid	GRM678	Himedia
EXpi 293F cells	100044202	ThermoFisher
Ficoll	26873-85-8	Sigma-Aldrich
Gel extraction kit	28706X4	Qiagen
Gelatin	G2500	Sigma-Aldrich
Glucose	MB037	Himedia

Glycerol	MB060	Himedia
Glycine	MB013	Himedia
HisPur™ Ni-NTA Magnetic Beads	88832	Thermo Scientific™
Histopaque	10771	Sigma-Aldrich
Hydrocortisone	RM556	Himedia
Hydrogen chloride	18-603-211	ThermoFisher
Imidazole	MB019-100G	Himedia
Isopropyl β-D-thiogalactoside	RM2578	Himedia
Kanamycin Sulphate	MB105	Himedia
L-glutamine	25030081	Gibco™
Ligase	15224017	Invitrogen™
Ligase buffer	46300018	Invitrogen™
LMB3 primer	Custom DNA oligos	Integrated DNA Technologies IDT
Luria Broth	M1245	Himedia
Luria Broth Agar	M1151-500G	Himedia
Magnesium chloride	MB237	Himedia
Magnesium sulphate	GRM1281	Himedia
Methanol	322415-250ml	Sigma-Aldrich
Mini prep kit	27106X4	Qiagen
Mono Sodium Phosphate	GRM3964	Himedia
NcoI-HF®	R3193S	New England Biolabs
Ni NTA beads	88221	ThermoFisher
NotI-HF®	R3189S	New England Biolabs
Penicillin	SD028	Himedia
PHEN primer	Custom DNA oligos	Integrated DNA Technologies IDT
Phosphate buffered saline	TS1101-20L	Himedia
Phytohemagglutinin (PHA)	10576015	Gibco™
Pierce™ Protein G Magnetic Beads	88848	Thermo Scientific™
Polybrene (Hexadimethrine bromide)	H9268	Sigma-Aldrich
Polyethylene glycol	MB149-500G	Himedia
Potassium Acetate	W292001	Sigma-Aldrich
Potassium chloride	P3911-25G	Sigma-Aldrich
Potassium dihydrogen phosphate	PO662-25G	Sigma-Aldrich
RNase A	EN0531	ThermoFisher
RPMI-1640 medium	11875093	Gibco™
Skim milk	GRM1254	Himedia
SnakeSkin™ Dialysis Tubing	88244	Thermo Scientific™
Sodium Azide	GRM1038	Himedia
Sodium bicarbonate	GRM849	Himedia
Sodium carbonate	GRM851	Himedia
Sodium chloride	MB023-1KG	Himedia

Sodium dodecyl-sulphate	0227-10G	VWR Life science
Sodium hydroxide	72064	Sigma-Aldrich
Sodium phosphate dibasic Bio Reagent	NIST2186II	Sigma-Aldrich
Stop solution	N600	Thermo Fisher Scientific
Streptomycin Sulphate	CMS220	Himedia
Sucrose	MB025	Himedia
TG1 Electrocompetent Cells	23227	Lucigen
TMB (Tetramethylbenzidine) Substrate solution	N301	Thermo Fisher Scientific
Tris base	TC072	Himedia
Tris Buffered Saline	R017R.0000	ThermoFisher
Tris free base	MB029-500G	Himedia
Tris-HCl	MB030	Himedia
Triton X100	MB031	Himedia
Trypan blue	T8154	Sigma-Aldrich
Trypsin	TC598	Himedia
Tween 20	MB067	Himedia

- Goat affinity purified Antibody to human IgG Fc, alkaline phosphatase conjugated goat affinity purified antibody to IgG Fc, and purified human IgG whole molecule were purchased from Cappel, MP Bio, USA.
- A human mAb 1418 against parvovirus B19 was gifted by Dr. Zolla Pazner, NYU SoM, USA

Plasticware

All plasticware used is disposable (glassware is not used in this work).

NAME	CATALOGUE NUMBER	COMPANY NAME
96 well flat bottom Immunol plate for ELISA	CLS3370	Corning
96 well round bottom Immunol plate for ELISA	CLS3367	Corning
96 well tissue culture plate	CLS 3628	Corning
Disposable pipettes of 5 mL, 10 mL and 20 mL	CLS4487	Corning
Microfuge tubes 1.5 mL	CLS3620	Corning
PCR tubes of 0.2 mL	PCR-02-A	Axygen
Petri dishes	460062	Tarson
Pipette tips 0.5–10 µL	AXYT300RS	Corning
Pipette tips 1–200 µL	CLS4860	Corning
T-25 cm^3 tissue culture flask	C6231	Corning
T-75 cm^3 tissue culture flask	C7231	Corning

BUFFERS

1. 10× stock of gel loading dye (see Recipes)
2. 2× Sample buffer (see Recipes)
3. Acrylamide:Bis (100 mL) (see Recipes)

4. Antibiotic concentration (see Recipes)
5. Buffer for Agarose Gel Electrophoresis (see Recipes)
6. Coating Buffer (see Recipes)
7. Composition of Reagents (see Recipes)
8. Counting of Expi293F™ Human Cells (see Recipes)
9. Destaining solution I (see Recipes)
10. Elution Buffer (1 L) (see Recipes)
11. Lower Gel Buffer [pH 8.8] 200 mL (see Recipes)
12. Lysis buffer (1 L) (see Recipes)
13. Phosphate buffered saline (see Recipes)
14. Purification of scFvs (see Recipes)
15. Tank Buffer 1× 2L (pH 8.3) (see Recipes)
16. Upper Gel Buffer [pH 6.8] 100 mL (see Recipes)
17. Wash Buffer (1 L) (see Recipes)
18. Wash Buffer (see Recipes)
19. Western Blotting solution (see Recipes)

Procedure

A. HELPER PHAGE PRODUCTION

A helper phage is necessary for transferring phagemid particles into *E. coli.* Phagemid particles contain (i) an antibiotic maker, (ii) antibody-G3P fusion protein, and (iii) phage origin of replication.
The phagemid libraries are amplified along with the antibody-G3P fusion protein and helper phage genes, which are required for infection, replication, assembly, and budding.

1. Take three Corning® 50 mL Falcon centrifuge tubes (FCTs) and label them A, B, and C.
2. Add 5 mL of 2× YT media to each FCT tube.
3. Take B and C as negative controls by adding ampicillin to one and kanamycin to the other.
4. Add 20 µL of TG1 *E. coli* cells to all three FCTs.
5. Incubate the FCTs at 37°C with shaking for overnight growth.
6. Subculture 20 µL of the previously inoculated TG1 cells in FCT A (Step A1) in 5 mL of fresh 2× YT media; incubate for 2–4 h at 37°C with shaking.
7. Then, add 40 µL of helper phage to the cultured TG1 cells.
8. Grow for 30 min at 37°C without shaking.
9. Grow for 30 min at 37°C with shaking.
10. Add this culture to 200 mL of fresh 2× YT media with kanamycin in a 50 µg/mL working concentration.
11. Allow to grow overnight at 30°C with shaking.
12. Remove the flask from the incubator, collect the culture in an autoclaved caesium bottle.
13. Centrifuge at 14,260 × *g* for 30 min at 4°C.
14. Pour the supernatant into another fresh caesium bottle and centrifuge at 14,260 × *g* and 4°C for 30 min.
15. Without disturbing the pellet, collect the supernatant in a glass bottle and add PEG/NaCl [20% (wt/vol) Polyethylene glycol 6000, 2.5 M NaCl], keeping the ratio of the supernatant and PEG/NaCl as 50:15.
16. Store in a cold room at 4°C for 4–5 h, or overnight for better results.
17. Spin the culture at 14,260 × *g* and 4°C for 1 h to allow the cells to settle down.
18. Without disturbing the pellet, discard the supernatant, and resuspend the pellet with 1× PBS, keeping it in 1.5 mL tubes.
19. Centrifuge the microtubes at 16,200 × *g* for 5 min. In case a pellet is formed, transfer the supernatant into fresh tubes and store at 4°C (Figure 1).

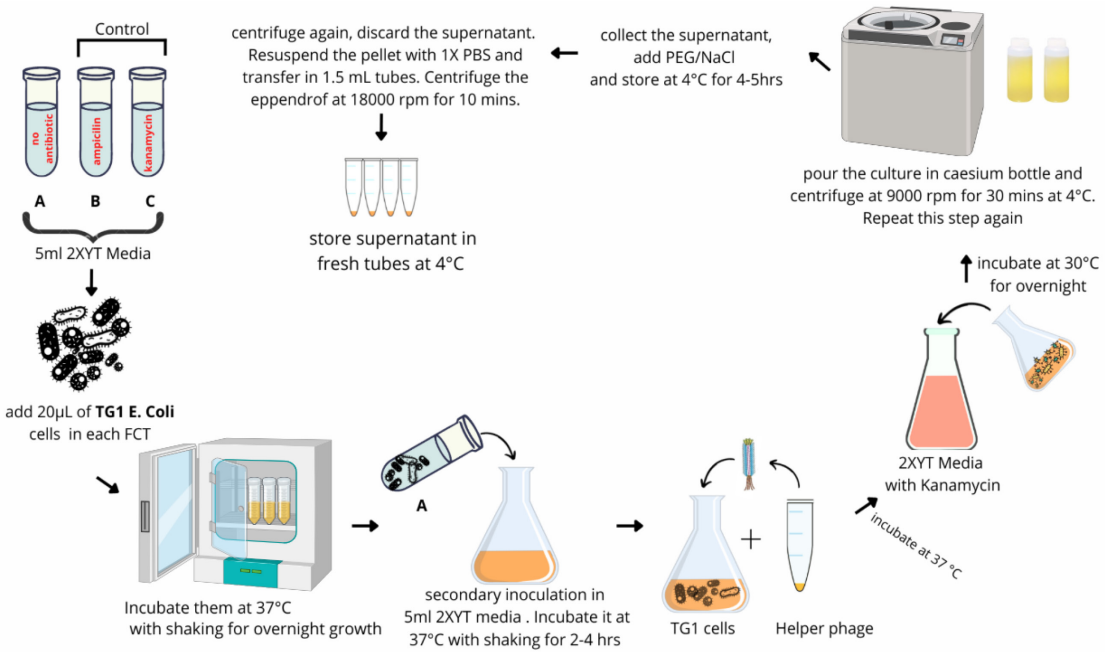


Figure 1. Schematic representation of the steps involved in Helper Phage preparation.

B. GROWING TOMLINSON I + J AND MAKING SECONDARY STOCK

1. Take 100 mL of 2× YT media containing ampicillin and 1% (vol/vol) glucose. To this media, add 500 µL of the Tomlinson I + J phage library stock.
2. Incubate for 1–2 h at 37°C with shaking, until the O.D. at 600 nm is approximately 0.4.
3. Divide the 100 mL of culture media into two parts. First, use 50 mL to grow the library; then, use the remaining media to make secondary stocks of the library.

Growing the library (phage stocks):

4. Take 50 mL of the 100 mL of grown media and add 200 µL of helper phage.
5. Incubate for 30 min at 37°C without shaking.
6. Centrifuge at 1,200 × *g* for 10 min.
7. Carefully discard the supernatant without disturbing the pellet.
8. Dissolve the pellet in 100 mL of 2× YT media containing 100 µg/mL ampicillin, 50 µg/mL kanamycin, and 0.1% (wt/vol) glucose.
9. Incubate the resuspended pellet overnight at 30°C with shaking.
10. After overnight incubation, transfer the culture to a centrifuge bottle (caesium bottle) and centrifuge at 14,260 × *g* and 4°C for 30 min.
11. Transfer the supernatant to another caesium bottle and centrifuge again at 14,260 × *g* and 4°C for 30 min.
12. Carefully transfer the supernatant into another autoclaved glass bottle and discard the pellet. Add PEG/NaCl (20% Polyethylene glycol 6000, 2.5 M NaCl) to the supernatant collected (15 mL of PEG/NaCl to 50 mL supernatant).
13. Store this in a cold room at 4°C for 4–5 h, or overnight for better results.
14. Spin the culture at 14,260 × *g* and 4°C for 1 h to allow the cells to settle down.
15. Without disturbing the pellet, discard the supernatant and resuspend the pellet with 1× PBS; keep it in 1.5 mL tubes.
16. Centrifuge the microtubes at 16,200 × *g* for 10 min. In case a pellet is formed, transfer the supernatant into fresh tubes and store at 4°C for short term storage. Add 15% (vol/vol) glycerol for longer storage at -80°C (Figure 2).

Making secondary stocks of phage library:

17. Grow the remaining 50 mL of media further for 2 h at 37°C with shaking.
18. Allow the cells to settle down by centrifuging the culture at 1,500 × *g* for 15 min.
19. Resuspend the pellet in 3 mL of 2× YT media containing 15% (vol/vol) glycerol.
20. Store at -80°C until further use (Figure 2).

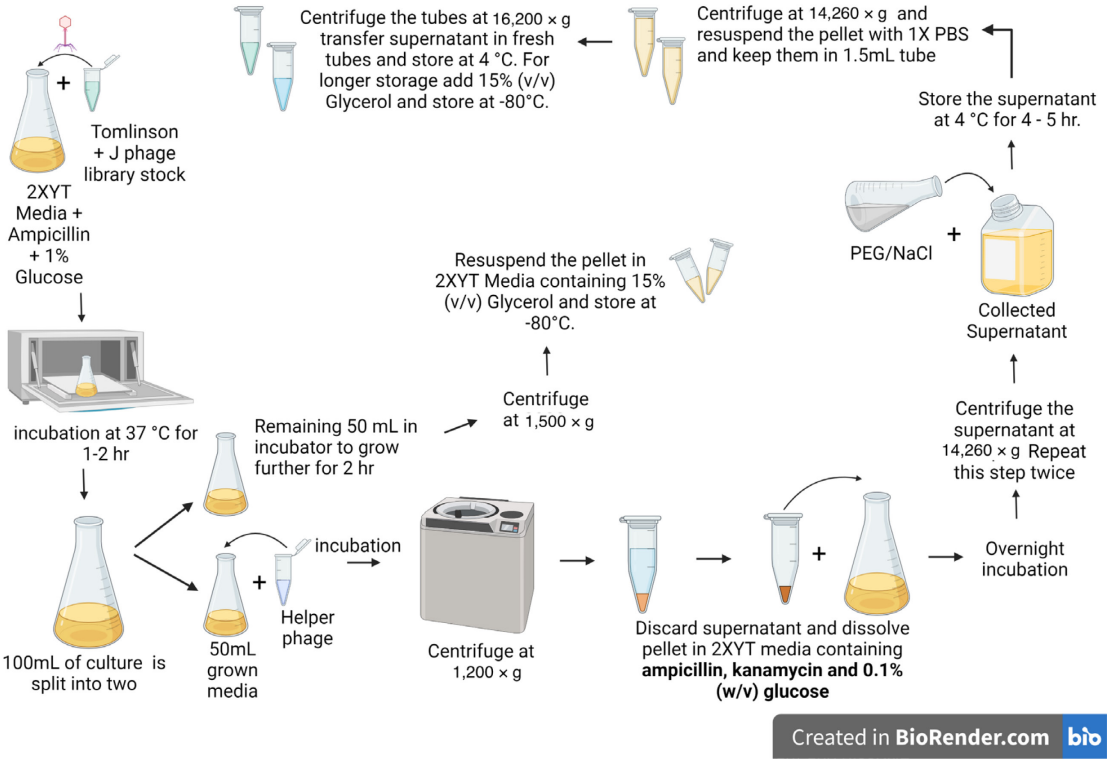


Figure 2. Schematic representation of the steps involved in library amplification for screening purposes and library secondary stock preparation.

C. BIO-PANNING

In this step, the target proteins are immobilized onto the surface of the microtiter plate. The first step is the addition of the rescued Tomlinson phage library. The second step involves binding, where the phage displaying scFvs, the highest affinity antibodies, bind the epitopes of the antigen, and those with low binding affinity are removed by washing. The antigen bound phage are eluted by enzymatic digestion using trypsin. The eluted phage are infected to TG1 followed by addition of helper phage for amplification. To accumulate phage displaying high-affinity antibody fragments, these steps were repeated three times with the amplified phage from the preceding round of panning, and each time, the number of washing cycles is increased.

ROUND 1

1. One day before the experiment, coat one row (say row C) of the ELISA plate, namely plate A, with the required antigen (100 µL per well), with a concentration of 5 µg. Coat the antigen with coating buffer.
2. Incubate plate A at 4°C overnight.
3. Next day, make 3%BSA in 5 mL of PBS and incubate for 10 min at 37°C. Then, coat another plate, *i.e.*, plate B; coat two rows (say rows C and D) and incubate the plate at 37°C for 1 h.
4. Plate C: coat one row (say row C) with a pinch of skim milk in 1 mL of autoclaved PBS + 700 µL of phage stock. Coat 100 µL per well.
5. Incubate plate C at room temperature for 30 min. Then, transfer the coating from row C to any other row,

say row D. Incubate for 30 min more.

6. After 1 h of coating plate B, wash the plate once with autoclaved PBS (250 µL per well) and transfer the content of plate C onto row C of plate B.
7. Again, incubate plate B at room temperature for 30 min and then transfer the content of row C onto row D, followed by another 30 min incubation.
8. While washing plate B, simultaneously wash plate A, by using autoclaved PBS (250 µL per well) and then block with 3% (wt/vol) BSA (200 µL per well). Incubate at room temperature for 1 h.
9. Wash plate A three times with autoclaved PBS.
10. Then, transfer plate B content onto plate A; followed by incubation for 1 h at room temperature.
11. After incubation, wash plate A with PBST 10 times. Make 1 mL of PBS containing 50 µL of trypsin and add 95 µL of this solution to each well.
12. Keep the plate for 10 min at 37°C.
13. Then, collect all the trypsinized content into one aliquot. This will be the output of bio-panning round 1.
14. Use the output of bio-panning 1 to calculate the transducing unit (TU) (Figure 3).

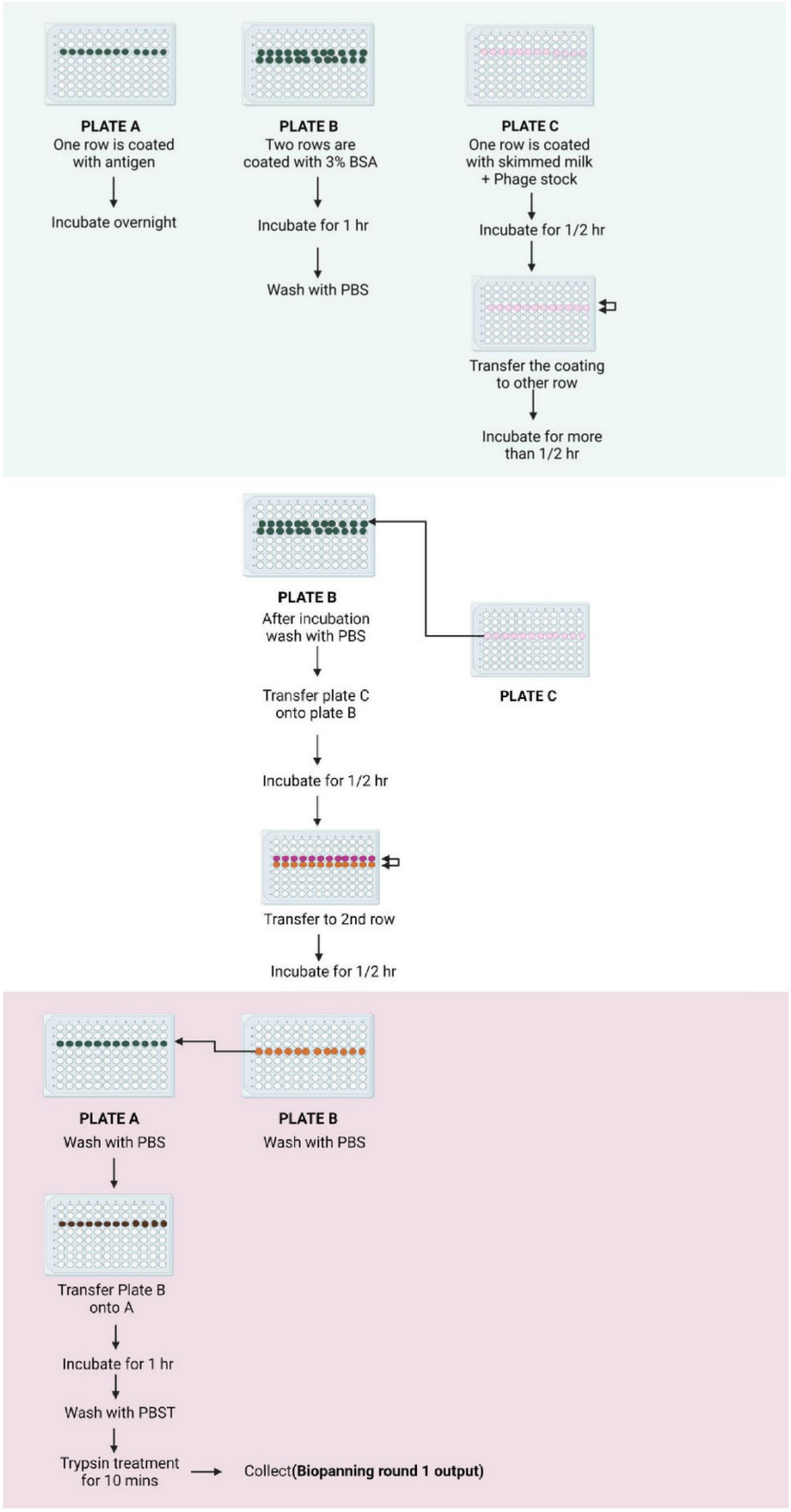


Figure 3. Schematic representation of the steps involved in the bio-panning process.

Preparation of the next round of bio-panning

15. Add 500 µL of bio-panning output into 5 mL of TG1 cell growth media.
16. Keep for 30 min at 37°C with shaking.
17. Centrifuge at 700 × *g* for 10 min.
18. Use 1 mL of supernatant to dissolve the pellet formed during centrifugation and throw the rest of the supernatant out.
19. Spread this on a bioassay dish containing 2× YT agar with ampicillin.
20. Allow the bacteria to grow at 37°C overnight.
21. Next day, add 3–5 mL of 2× YT media containing 15% glycerol onto the bioassay dish and scrape all the colonies grown overnight. Collect them in a fresh tube.
22. Use 100 µL of the scraped colonies and store the rest.
23. Add 100 µL of scraped colonies to 50 mL of 2× YT media containing ampicillin (100 µg/mL) and 1% (vol/vol) glucose.
24. Allow it to grow for 2 h at 37°C with shaking.
25. Take 10 mL of the above culture and add a 40 µL of helper phage. Incubate for 30 min at 37 °C without shaking.
26. Centrifuge the culture at 700 ×g for 15 min and discard the supernatant without disturbing the pellet.
27. Dissolve the pellet in 50 mL of 2× YT media containing 100 µg/mL ampicillin, 50 µg/mL kanamycin, and 0.1% glucose. Incubate overnight at 30°C with shaking.
28. Centrifuge the overnight grown culture at 14,260 × *g* and 4°C for 30 min, collect the supernatant in a fresh cesium bottle and centrifuge again at 14,260 × *g* and 4°C for 30 min.
29. Carefully transfer the supernatant into an autoclaved glass bottle and discard the pellet. Add PEG/NaCl (20% Polyethylene glycol 6000, 2.5 M NaCl) to the supernatant collected (15 mL of PEG/NaCl to 50 mL of supernatant).
30. Store this in a cold room at 4°C for 4–5 h, or overnight for better results.
31. Spin the culture at 14,260 × *g* for 1 h at 4°C to allow the cells to settle down.
32. Without disturbing the pellet, discard the supernatant and resuspend the pellet with 1× PBS, keeping it in 1.5 mL tubes.
33. Centrifuge the microtubes at 16,200 × *g* for 10 min. In case a pellet is formed, transfer the supernatant into fresh tubes and store it at 4°C.
34. The collected phage is to be used as an input in the next round of bio-panning by coating this on plate C instead of phage stock.

Note: With each bio-panning round, decrease the antigen coating concentration (for example, round 1 with 5 µg/µL, round 2 with 3 µg/µL, and round 3 with 1.5 µg/µL) and calculate the TU of every input and output used during phage selection.

D. SCREENING BY PHAGE ELISA

Day 0: One day before performing ELISA

1. For the phage selection process, grow the colonies of the last bio-panning round output. Inoculate the colonies formed during the last round of bio-panning output; each inoculation is done in 5 mL of 2× YT media containing 100 µg/mL ampicillin.
2. Incubate for 30 min at 37°C without shaking. Wait until OD_{600} of the culture reaches 0.4–0.6.
   Back up set: At this step, take 200 µL of the culture of each clone and add 200 µL of autoclaved 50% (vol/vol) glycerol solution to make a stock and store at -80°C for future experiments
3. Add 20 µL of helper phage.
4. Incubate again for 30 min at 37°C with shaking.
5. Add 50 µg/mL of kanamycin and incubate overnight at 30°C with shaking at 140–160 × *g*.
6. Coat the 96 well assay plate with the required antigen with a concentration of 2 µg/µL. Do BSA coating as negative control and keep the plate overnight at 4°C (Figure 4).

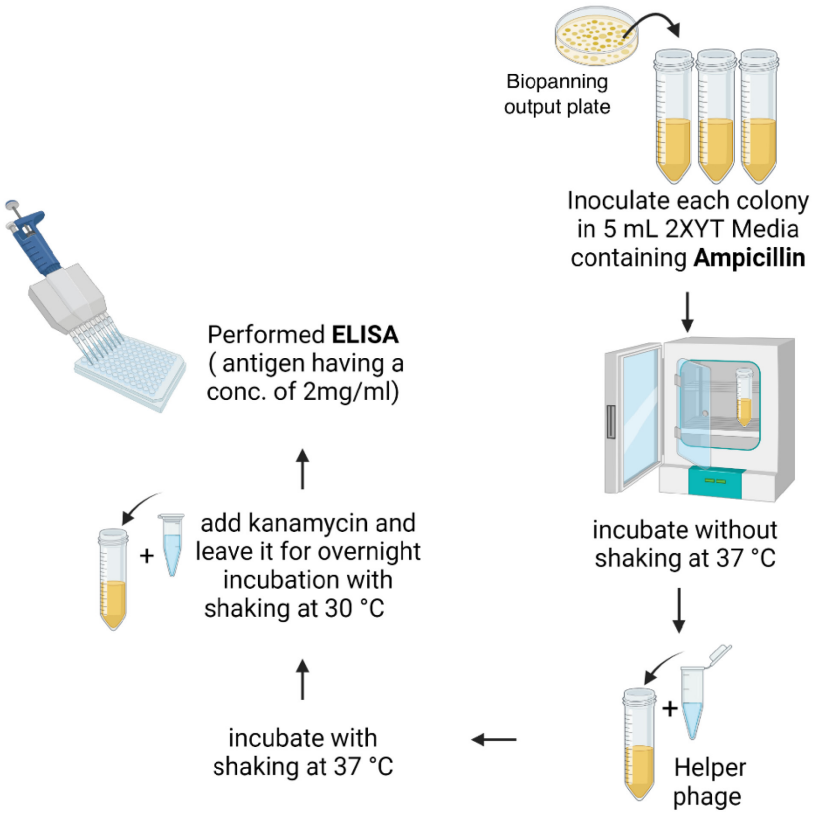


Figure 4. Schematic representation of the steps involved in the Phage ELISA screening process.

ELISA

7. Next day, take the antigen-coated plate out of 4°C and wash once with PBS.
8. Block the plate with 200 µL of 5% skim milk in PBS per well.
9. Incubate the plate for 1 h at room temperature.
10. Take all the colony inoculation out of the incubator and centrifuge the tubes at 3,900 × *g* for 20 min.
11. After 1 h blocking, wash the ELISA plate three times with PBS (250 µL).
    Note: Blocking can be extended to 90 min if required, based on the timing of the parallel steps
12. After 1 h of incubation, wash the plate three times with PBS.
13. Then add 100 µL of primary antibody per well, *i.e.*, 50 µL of supernatant from all the centrifuged tubes and 50 µL of skim milk (diluted in 1:1).
14. Incubate for 1 h at room temperature.
15. Wash the plate with 250 µL of 0.1% PBST four times.
16. Add 100 µL of secondary antibody per well (1:2,000). Follow with a 1 h incubation at room temperature in the dark.
17. Wash the plate with 250 µL of 0.1% PBST six times.
18. Add 100 µL of the substrate (TMB) to each well.
19. Allow the reaction to take place for 15–20 min in the dark.
    To stop the reaction, add 50 µL of stop solution (2 NH_2SO_4) per well, and read the plate at 450 nm on a multimode ELISA reader.

E. ISOLATION OF PLASMID DNA

1. Identify the positive binding clones in Phage ELISA (at least four times more than the negative control).
2. Take two FCTs and label them A and B; add 5 mL of 2× YT media to each FCT.
3. Take B and add ampicillin (100 µg/mL) and C as negative control by adding kanamycin (50 µg/mL) in it.

4. In FCTs A and B, inoculate from a glycerol stock that is preserved at -80°C.
5. Incubate the FCT at 37°C with shaking for overnight growth.
6. Next morning, check tubes A and B. Tube A culture should be turbid, and in tube B, there should be no growth.
7. Spin down by centrifuging the culture at 2,820 × *g* for 15 min.
8. For plasmid isolation, use the Qiagen Miniprep kit following the manufacturer's instructions.
9. Check the quality and concentration of the isolated plasmid DNA using a nanodrop spectrophotometer. The 260/280 ratio of the isolated DNA should be 1.8.
10. Prepare a 0.8% Agarose gel and check the quality of the isolated plasmid DNA on the gel.
11. Make a 10 µL aliquot of the plasmid DNA, and use LMB3 and PHEN sequencing primers for sequencing the scFv insert sequence.

Soluble ELISA

12. Perform ELISA as described in the phage ELISA section.
13. Add 100 µL of purified scFv and incubate for 1 h at room temperature.
14. Wash the plate three times with 0.1% PBST.
15. Use a 1:1,000 dilution of primary antibody (anti-His tag) in 2% MPBS and incubate at room temperature.
16. Wash three times with 0.1% PBST.
17. Use 1:2,000 diluted anti-rabbit-HRP conjugated secondary antibody in 2% MPBS and incubate at room temperature, followed by washing, as mentioned above.
18. Add 100 µL of TMB substrate and allow the color to develop. Once the color appears, add 8 N H_2SO_4 to stop the reaction.
19. Read the plate at 450 nm in ELISA reader.

F. DILUTION AND PLATING FOR TRANSDUCING UNIT (TU) CALCULATION

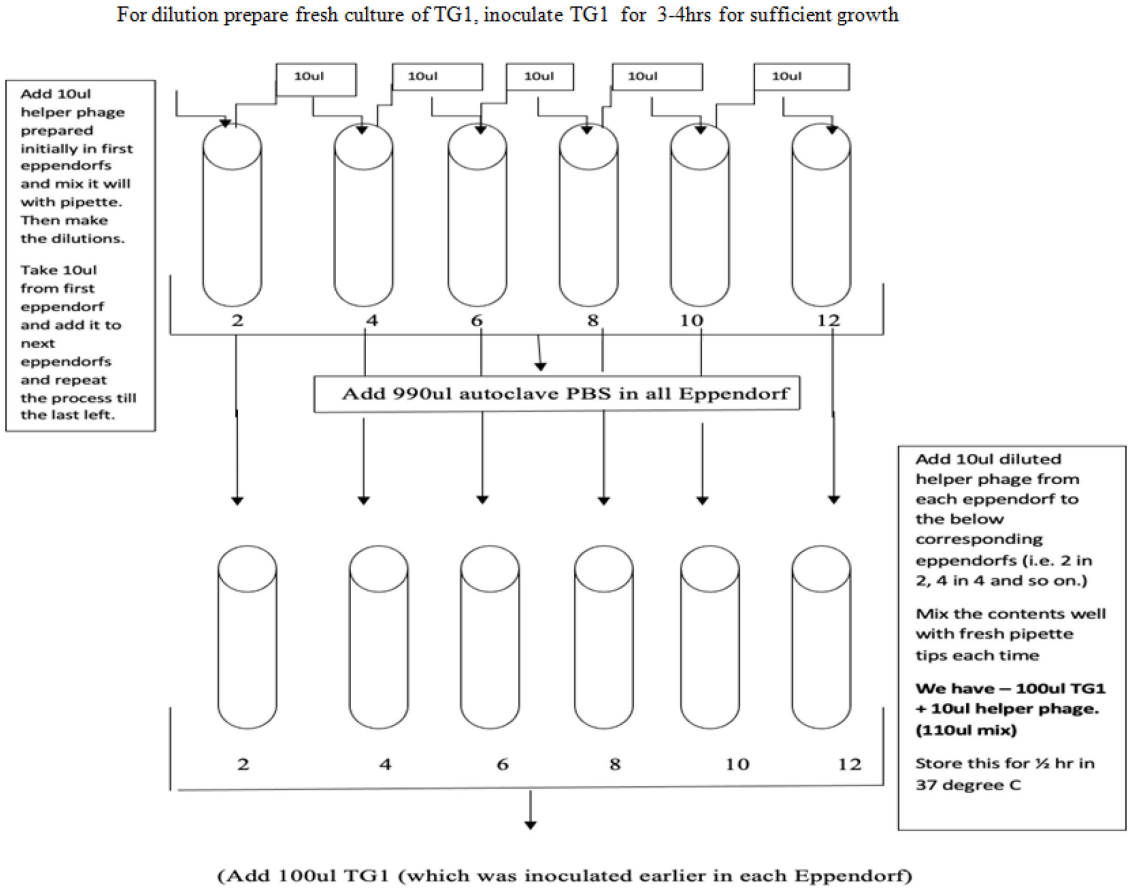


Figure 5. Representative image showing dilution preparation strategy for helper phage/library TU calculation.

1. The plating of each dilution is done on a different plate containing ampicillin.
2. Pour the mixture of phage and bacteria on a plate and spread slowly using a spreader. Label each plate with the dilution that it contains.
3. Incubate the plates at 37°C for overnight growth. Next day, count the colonies for TU calculation (Figure 5).

G. CALCULATE TRANSDUCING UNIT

TU = (No. of colony × 1000)/(10 × dilution). For a 10^{-8} dilution plate, we got nine colonies;
Then the TU is calculated as: $(9 \times 1000)/(10 \times 10^{-8}) = 9 \times 10^{10}$

H. PREPARATION OF COMPETENT CELLS

1. Streak *E. coli* TG1 cells on an LB plate and allow cells to grow at 37°C overnight.
2. Inoculate a single colony in 5 mL of LB media and grow overnight at 37°C.
3. Subculture in 100 mL of LB by inoculating 1 mL of an overnight culture of *E. coli,* and grow at 37°C on a shaker until the O.D. at 600 nm reaches approximately 0.6.
4. Cool culture on ice immediately, and harvest cells by centrifugation at 4,200 × *g* and 4°C for 5 min.
5. Remove the supernatant carefully; remove any traces of supernatant by inverting the centrifuge tube on paper towels.
6. Resuspend the bacterial pellet in 10 mL of ice-cold 0.1 M $CaCl_2$ (autoclaved) and incubate on ice for 30 min.
7. Recover cells by centrifugation as described above, resuspended in 5 mL of 0.1 M $CaCl_2$, and aliquot 200 µL of cells in microcentrifuge tubes.

I. VECTOR DESIGN

A representative strategy for vector design is shown in Figure 6.
A set of designed PCR primers to replace TAG amber codon into TAA in the junction of the scFv-pIII junction is used for vector construction.
Forward Primer: 5'CACATCATCATCACCATCACGGGTAATAAGAACAAAAACTCATCTC3'
Reverse primer: 5'GAGATGAGTTTTTGTTCTTATTACCCGTGATGGTGATGATGATGTG3'.
PCR Reaction setup:

Steps	**Initial Denaturation**	**Cycling 16×**			**Extension**	**Hold**
Temperature	95°C	95°C	52°C	72°C	72°C	10°C
Time	2 min	30 s	50 s	4 min	5 min	∞

The PCR product is digested by *Dpn*1 enzyme

PCR product	**20 µL**
***Dpn*1 enzyme**	1 µL
Cut smart buffer	2.5 µL

Incubate the reaction mixture at 37°C for 2 h. Tap the tube in between.

1. Thaw two vials of competent cells from the -80°C freezer on ice for 5–10 min. DO NOT tap at this step.
2. Add 5 µL of *Dpn*1 digested product into one tube, and keep the other tube as blank or negative control. Incubate the cells on ice for 30 min.
3. After 30 min, place both the tubes in the floater to heat shock for 60 s (at this step, set water bath at 42°C).

4. Immediately place the tubes in ice for 5 min; at this step, pre-warm the media at 37°C.
5. Add 900 µL of pre-warmed 2× YT medium to the cells in the tube.
6. Incubate the tubes in the shaker for 60 min at 220 rpm to grow the cells.
7. Take out the tubes from the shaker, aspirate 100 µL of cell suspension, and plate/spread on LB-Agar-ampicillin plates. Incubate plates in a 37°C incubator for 16 h or overnight.
8. Next morning, take out the plates, count the colonies, and store at 4°C until further use.
9. Add 5 mL of 2× YT supplemented with a standard concentration of ampicillin to five tubes. Pick a single colony for inoculation and culture of the colony obtained on the plates; incubate at 37°C with 220 rpm shaking. Incubate also one tube of media as a control.
10. Next morning, isolate the plasmid DNA from all four tubes using the Qiagen plasmid isolation kit as per the manufacturer's instructions.
11. Check the quality of the isolated plasmid using a spectrophotometer by observing the 260/280 DNA ratio.
12. Aliquot the DNA and send these samples for sequencing using vector-specific primers.
13. Analyze the DNA sequence data for point mutations and mark the positive clones for further use. Discard the negative clones.

Digestion of designed vector

Reaction mixture for restriction digestion:

Component	Volume (µL)
Plasmid DNA (250 ng·µL^{-1})	10 µL
10× CutSmart Buffer (NEB)	5 µL
*Nco*I-HF (NEB)	2 µL
*Not*I-HF (NEB)	2 µL
Nuclease free water	31 µL

Incubate the reaction at 37°C for 2 h.

1. After 2 h, add 10 µL of 5× gel loading dye and run the sample on a 0.8% Agarose gel, at 100 V for approximately 60 min.
2. Cut and excise the digested vector DNA from the agarose gel using a sharp surgical blade. Place the excised fragment in a 1.5 mL Eppendorf tube. Purify the digested vector from the excised DNA using the Qiagen Gel extraction kit as per the manufacturer's protocol.
3. Evaluate the quality of the purified digested vector DNA using the spectrophotometer by calculating the 260/280 ratio. This purified vector is used for future cloning reactions.

Digestion of scFv clones

The phage ELISA binding positive clone that showed no binding in soluble ELISA was further used to isolate plasmid DNA from single colonies, as described in this section.

The digestion reaction is set up as described below:

Component	Volume (µL)
Plasmid DNA (250 ng·µL^{-1})	10 µL
10× CutSmart Buffer (NEB)	5 µL
*Nco*I-HF (NEB)	2 µL
*Not*I-HF (NEB)	2 µL
Nuclease free water	31 µL

1. After 2 h add 10 µL of 5× gel loading dye and run the sample on a 1% Agarose gel, at 100 V for approximately 40–60 min.

2. Two bands should be observed in the agarose gel, one band size of approximately 4 kb corresponding to vector DNA and another of 800 bp corresponding to scFv DNA.
3. Excise the agarose gel slice containing the relevant DNA Fragments (scFv insert 800 bp) and remove extra agarose to minimize the gel slice.
4. Transfer the gel slice into a microcentrifuge tube and purify using the Qiagen gel extraction kit as per the manufacturer's instructions.
5. Use this purified scFv DNA for cloning into a newly designed vector.

Cloning of scFv DNA into newly designed vector

Component	Volume (µL)
scFv DNA insert	10 µL
Designed vector	5 µL
10× Ligase buffer	2 µL
Ligase	2 µL
Nuclease free water	31 µL

1. Incubate the ligation reaction mixture at room temperature for 4–6 h. Alternatively, this can be kept at room temperature overnight.
2. After 4–6 h incubation, thaw the TG1 competent cells on the ice for 5 min.
3. Add 5 µL of ligation mixture to one tube and keep the second tube without insert and/or ligation mixture as a negative control.
4. Heat-shock the cells for 60 s and immediately keep on ice for 5 min.
5. Add 900 µL of 2× YT medium and incubate the tubes in a shaker incubator at 37°C for 1 h.
6. After 1 h, centrifuge the tube at 2,400 × *g* for 5 min. Decant the supernatant and resuspend the pellet in reaming leftover media. Plate this on pre-warmed 2× YT-Agar plates supplemented with ampicillin, or optionally you can use LB-Agar plates with ampicillin.
7. Incubate the plates in a 37°C incubator overnight or 12–16 h.
8. Next morning, count the colonies on the reaction plate. NO COLONIES should be there in the control plate; otherwise, repeat the experiment with all new and freshly prepared reagents.
9. Inoculate the single colony in 5 mL of 2× YT containing a standard concentration of ampicillin in two 50 mL FCTs. Label one tube as reaction and the other as blank, and incubate both tubes in a shaker incubator (200 to 220 rpm) at 37°C overnight.
10. Transfer a small inoculum (approximately 4 mL) from the overnight primary culture to a 2 L flask (400 mL media) 2× TY containing 100 µg/mL ampicillin and 0.1% glucose. Grow shaking (250 rpm) at 37°C until the OD_{600} is approximately 0.9 (approximately 3 to 3.5 h).
11. Once the OD of the culture reaches 0.9, add isopropyl β-D-thiogalactoside at a final concentration of 1 mM IPTG. Continue shaking (250 rpm) at 30°C overnight.
12. Harvest the cells by centrifugation at 4,000 × *g* for 15 min. Store the cell pellet at -20°C if desired or process immediately.

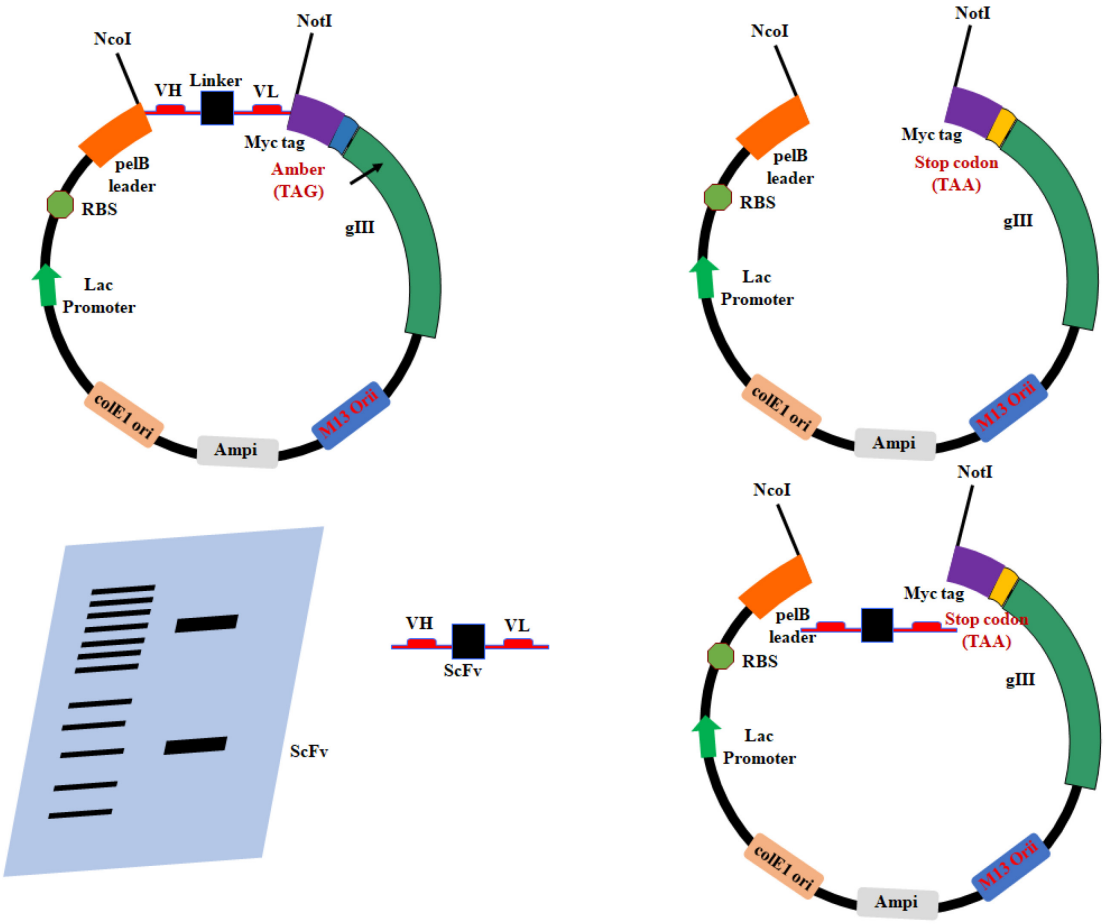


Figure 6. Schematic representation of the modified vector design strategy.
Diagram depicting a modified method for soluble expression of scFv genes with amber stop codons. The amber stop codon (TAG) between the scFv-pIII gene in the original vector is altered to TAA, which prohibits the creation of scFv-pIII fusion proteins. The scFv genes are cloned directly into the modified vector using the same restriction site Nco1/Not1 that is used to clone the scFv gene into the original vector (Perween *et al*., 2021a).

Resuspension of cell pellet and cell extract preparation

a. Adjust pH to 8.0 using NaOH.
b. Resuspend the pellet in 30 mM Tris-HCl, 20% (wt/vol) sucrose, pH 8.0, at 80 mL/gram of wet weight. Incubate on ice and add 500 mM EDTA dropwise to a final concentration of 1 mM; then incubate the cells on ice for 20 min with gentle agitation.
c. Clarify the cell suspension by centrifuging at 8,000 × *g* and 4°C for 20 min.
d. Collect the supernatant and resuspend the cells in the same volume of ice-cold 5 mM $MgSO_4$ and incubate on ice for 20 min with gentle agitation.
e. Centrifuge the cells at 8,000 × *g* and 4°C for 20 min. Collect the supernatant (supernatant is osmotic shock fluid containing periplasmic proteins) and dialyze extensively against lysis buffer.
f. Filter the dialyzed supernatant through a 0.2 µm filter before continuing with the purification. Equilibrate the resin with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, and pH = 8.0) prior the use of the Ni++ ions.

Purification OfscFvs

a. For purification, use Ni-NTA beads; add 5 mL of 50% slurry of Ni-NTA-Agarose resin in a purification column and allow the beads to settle down.

b. Wash the beads using MilliQ (MQ) water; add 10 column volume (CV) of MQ.
c. Use 10 CV of binding buffer (20 mM Tris; pH = 7.2, 500 mM NaCl and 10 mM Imidazole) to equilibrate the column. Thus, the same pH and buffer composition as that of the Ni++ ion resin ensuring that the sample binds properly
d. Add the filtered supernatant into the column and allow it to pass through gravitational force.
e. After the supernatant has been passed, wash the column to remove any impurity. Add 20–30 CV of washing buffer (20 mM Tris; pH = 7.2, 500 mM NaCl, and 25 mM Imidazole).
f. Elute the bound protein from beads, and use 5–6 CV of elution buffer (20 mM Tris pH = 7.2, 500 mM NaCl, and 500 mM Imidazole). Analyze the protein quality in terms of purity on a 15% Tri-glycine SDS-PAGE.
g. Dialyze the protein using activated dialysis tubing, clip both the ends of the tube tightly, and put it in a beaker containing cold PBS. With the help of a magnetic stirrer, allow it to spin overnight at 4°C in a cold room.
h. Mount the Superdex75 Increase column in AKTA FPLC system. Wash the pump thoroughly with PBS.
i. Use 2 CV of PBS to equilibrate the column; load approximately 500 µL of the purified concentrated protein through the loop with 0.5 mL of fraction volume.
j. Collect the different factions and analyze through SDS PAGE. Store the protein at -80°C in aliquots for further use. Mix 30 µL of sample with 5× SDS gel loading dye (6 µL). Heat the sample for 10 min at 100°C before loading.

Recipes

1. Lysis buffer (1 L)
50 mM NaH_2PO_4 (6.90 g of $NaH_2PO_4 \cdot H_2O$; MW 137.99 g mol^{-1})
300 mM NaCl (17.54 g of NaCl; MW 58.44 g mol^{-1})
10 mM Imidazole (0.68 g of Imidazole; MW 68.08 g mol^{-1})
Adjust pH to 8.0 using NaOH.

2. Wash Buffer (1 L)
50 mM NaH_2PO_4 (6.90 g of $NaH_2PO_4 \cdot H_2O$; MW 137.99 g mol^{-1})
300 mM NaCl (17.54 g of NaCl; MW 58.44 g mol^{-1})
30 mM Imidazole (2.04 g of Imidazole; MW 68.08 g mol^{-1})
Adjust pH to 8.0 using NaOH.

3. Elution Buffer (1 L)
50 mM NaH_2PO_4 (6.90 g of $NaH_2PO_4 \cdot H_2O$; MW 137.99 g mol^{-1})
300 mM NaCl (17.54 g of NaCl; MW 58.44 g mol^{-1})
300 mM Imidazole (20.4 g of Imidazole; MW 68.08 g mol^{-1})

4. Buffer for Agarose Gel Electrophoresis
TBE (Tris Boric acid EDTA) Buffer, pH = 8.0
For 10× TBE Buffer, dissolve 108 g Tris base, 55 g Boric acid, and 7.4 g of EDTA in 750 mL of water. Adjust the pH of the solution to 8.0 and make the final volume up to one liter. 1× TBE buffer is used as the working solution.

5. Wash Buffer
PBS-Tween-20, pH = 7.4
200 mL of 10× PBS
1 mL of Tween 20
Adjust volume to 2 L with MQ water

6. Coating Buffer

Carbonate Buffer, pH = 9.6
1.59 g Na_2CO_3
2.93 g $NaHCO_3$
0.2 g NaN_3
Adjust volume to 1 L with MQ water

7. Counting of Expi293F™ Human Cells

a. Aspirate 0.1 mL of cell suspension from the single cell suspension and stain with 0.1 mL of trypan blue (0.4%).
b. Count both Dead and live cells for percentage viability calculations.
c. Calculations:
Cell counting = No. of viable cells × 2 × 10^4 Cells per mL

8. 10× stock of gel loading dye (10 mL)

a. Weigh 25 mg of bromophenol blue and dissolve in 7 mL of ddH_2O in a 30 mL screwcap tube.
b. Add 2.5 g of Ficoll and dissolve it (keep in shaker overnight to allow it to dissolve completely).
c. Measure the volume using a pipette and make up to 10 mL using sterile ddH_2O. Label and store at 4°C.
d. The final concentration would be 0.25%.
e. Bromophenol blue and 25% Ficoll.

9. Antibiotic concentration

a. Stock concentration= 100 mg/mL
b. Working concentration= 100 µg/mL
c. Therefore, for 2 mL
d. N1V1=N2V2
e. X*100000 = 200*100
f. X = 200 µL

10. Phosphate buffered saline [pH 7.2]

- NaCl = 8.0 g
- KCl = 0.2 g
- Na_2HPO_4 = 1.15 g
- KH_2PO_4 = 0.2 g

All the components were dissolved in 700 mL of distilled water, and the pH was checked to be at 7.2 and made up to 1,000 mL.

11. Acrylamide:Bis (100 mL, 30% stock)

a. Acrylamide = 29.2 g
b. Bis acrylamide = 0.8 g
c. ddH_2O = 7 mL

Acrylamide and Bis acrylamide were weighed and dissolved in 70 mL of water, and the volume was made up to 100 mL. The solution was filtered through Whatman No.2 paper and stored at 4°C in an amber bottle.

12. Upper Gel Buffer [pH 6.8] 100 mL

This is nothing but the stacking gel buffer, *i.e.*, 0.5 Molar Tris-HCl (4×). 6.6 g of Tris base was dissolved in 70 mL of ddH_2O, and pH was checked and adjusted to 6.8 using 5 N HCl. The volume was made up to 100 mL and stored at 4°C.

13. Lower Gel Buffer [pH 8.8] 200 mL

Separating gel buffer (1.5 M Tris-HCl (4×))
36.3 g of Tris was dissolved in 125 mL of ddH_2O. pH was adjusted to 8.8 using 5 N HCl. Volume was made up to 200 mL and autoclaved. The solution was stored at 4°C.

14. Tank Buffer 1× (pH 8.3) 2 L

Tris base = 6.0 g Final conc. 0.025 M
Glycine = 28.8 g Final conc. 0.192 M
SDS = 2 g Final conc. 0.1 M
ddH_2O = 1,750 mL
pH was checked to be approximately 8.3 and volume was made up to 2 L.

15. Destaining solution I

Methanol = 250 mL (50%)
Acetic acid = 35 mL (7%)
ddH_2O= 215 mL

16. 2× Sample buffer (5 mL)

- Tris-HCl (pH 6.8) = 1.25 mL (0.12 M)
- SDS = 0.2 g (4%)
- BME = 500 µL (10%)
- Glycerol = 1 mL (20%)
- Bromophenol blue (0.15%) = 500 µL (0.015%)
- ddH_2O = 1.75 mL
- Total = 5 mL

This was dissolved into aliquots and frozen in -20°C [0.15% Bromophenol Blue is prepared by dissolving 15 mg in 0.2 mL methanol and then adding 9.8 mL of water].

17. Western Blotting solution Preparation

Transfer Buffer
Tris = 0.6 g (0.025 M)
Glycine = 2.88 g (0.192 M)
Methanol = 40 mL (20%)
SDS = 60 mg (0.03%)
Volume was made upto 160 mL and autoclaved. To this, 40 mL of methanol was added and stored at 4°C.

TBS (Tris Buffer Saline) (400 mL)
Tris = 4.84 g (0.1 M)
NaCl = 3.6 g (0.9%)
Components were dissolved, pH was adjusted to 7.5 with HCl, and volume was made up to 400 mL.

TTBS (250 mL)
TBS = 250 mL
Tween 20 = 250 µL

Blocking solution (30 mL)
TTBS = 12 mL
5% Gelatin = 18 mL (3%)

Composition of Reagents:

Buffer P1 [Resuspension buffer]
50 mM Tris-HCl (pH 8)
10 mM EDTA
100 µL/mL RNase A

Buffer P2 [Lysis buffer]
200 mM NaOH
1% SDS (w/v)

Buffer P3 [Neutralization buffer]
3 mM potassium acetate (pH 5.5)

Buffer QBT [Equilibrium buffer]
750 mM NaCl
50 mM Mops (pH 7)
15% isopropanol (v/v)
0.15% Triton X-100 (v/v)

Buffer QC [Wash buffer]
1 mM NaCl
50 mM Mops (pH 7)
15% isopropanol (v/v)

Acknowledgments

We thank the Medical Research Council of the United Kingdom for allowing us to use the Tomlinson libraries. We thank Prof. S Sinha, department of Biochemistry, AIIMS, New Delhi for his critical inputs during screening of the libraries. information—This work was supported by the Department of Biotechnology and by a Translational Health Science & Technology Institute core grant.

Competing interests

There are no conflicts of interest or competing interests.

References

Barderas, R., Shochat, S., Martinez-Torrecuadrada, J., Altschuh, D., Meloen, R. and Ignacio Casal, J. (2006). A fast mutagenesis procedure to recover soluble and functional scFvs containing amber stop codons from synthetic and semisynthetic antibody libraries. *J Immunol Methods* 312(1-2): 182-189.

Borghardt, J. M., Kloft, C. and Sharma, A. (2018). Inhaled Therapy in Respiratory Disease: The Complex Interplay of Pulmonary Kinetic Processes. *Can Respir J* 2018: 2732017.

Frenzel, A., Schirrmann, T. and Hust, M. (2016). Phage display-derived human antibodies in clinical development and therapy. *MAbs* 8(7): 1177-1194.

Kumar, R., Andrabi, R., Tiwari, A., Prakash, S. S., Wig, N., Dutta, D., Sankhyan, A., Khan, L., Sinha, S. and Luthra, K. (2012). A novel strategy for efficient production of anti-V3 human scFvs against HIV-1 clade C. *BMC Biotechnol* 12: 87.

Kumar, R., Kumari, R., Khan, L., Sankhyan, A., Parray, H. A., Tiwari, A., Wig, N., Sinha, S. and Luthra, K. (2019a). Isolation and Characterization of Cross-Neutralizing Human Anti-V3 Single-Chain Variable Fragments (scFvs) Against HIV-1 from an Antigen Preselected Phage Library. *Appl Biochem Biotechnol* 187(3): 1011-1027.

Kumar, R., Parray, H., Narayan, N., Garg, S., Rizvi, Z. A., Shrivastava, T., Kushwaha, S., Singh, J., Murugavelu, P., Anantharaj, A., *et al*. (2022). A broadly neutralising monoclonal antibody overcomes the mutational landscape of emerging SARS-CoV2 variant of concerns. *Research Square*. DOI: 10.21203/rs.3.rs-1431974/v1.

Kumar, R., Parray, H. A., Shrivastava, T., Sinha, S. and Luthra, K. (2019b). Phage display antibody libraries: A robust approach for generation of recombinant human monoclonal antibodies. *Int J Biol Macromol* 135: 907-918.

Marcus, W. D., Lindsay, S. M. and Sierks, M. R. (2006). Identification and repair of positive binding antibodies containing randomly generated amber codons from synthetic phage display libraries. *Biotechnol Prog* 22(3): 919-922.

Parray, H. A., Shukla, S., Samal, S., Shrivastava, T., Ahmed, S., Sharma, C. and Kumar, R. (2020). Hybridoma technology a versatile method for isolation of monoclonal antibodies, its applicability across species, limitations, advancement and future perspectives. *Int Immunopharmacol* 85: 106639.

Perween, R., Ahmed, S., Shrivastava, T., Parray, H. A., Singh, B., Pindari, K. S., Sharma, C., Shukla, S., Sinha, S., Panchal, A., K. *et al.* (2021a). A rapid novel strategy for screening of antibody phage libraries for production, purification, and functional characterization of amber stop codons containing single-chain antibody fragments. *Biotechnol Prog* 37(3): e3136.

Reader, R. H., Workman, R. G., Maddison, B. C. and Gough, K. C. (2019). Advances in the Production and Batch Reformatting of Phage Antibody Libraries. *Mol Biotechnol* 61(11): 801-815.